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ORIGINAL ARTICLE

A single transgene locus triggers both transcriptional and post-transcriptional silencing through double-stranded RNA production

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Abstract Silencing of a target locus by an unlinked silencing locus can result from transcription inhibition (transcriptional gene silencing; TGS) or mRNA degradation (post-transcriptional gene silencing; PTGS), owing to the production of double-stranded RNA (dsRNA) corresponding to promoter or transcribed sequences, respectively. The involvement of distinct cellular components in each process suggests that dsRNA-induced TGS and PTGS likely result from the diversification of an ancient common mechanism. However, a strict comparison of TGS and PTGS has been difficult to achieve because it generally relies on the analysis of distinct silencing loci. We describe a single transgene locus that triggers both TGS and PTGS, owing to the production of dsRNA corresponding to promoter and transcribed sequences of different target genes. We describe mutants and epigenetic variants derived from this locus and propose a model for the production of dsRNA. Also, we show that PTGS, but not TGS, is graft-transmissible, which together with the sensitivity of PTGS, but not TGS, to RNA viruses that replicate in the cytoplasm, suggest that the nuclear compartmentalization of TGS is responsible for cell-autonomy. In contrast, we contribute local and systemic trafficking of silencing signals and sensitivity to viruses to the cytoplasmic steps of PTGS and to amplification steps that require high levels of target mRNAs.

Keywords Double-stranded RNA · Post-transcriptional gene silencing · Short interfering RNA · Systemic silencing · Transcriptional gene silencing

Abbreviations

dsRNA	Double-stranded RNA
GUS	ß-Glucuronidase
NIR	Nitrate reductase
PTGS	Post-transcriptional gene silencing
siRNA	Short interfering RNA
ssRNA	Single stranded RNA
TGS	Transcriptional gene silencing

Introduction

Homology-dependent gene silencing (HDGS) is a phenomenon that consists of the silencing of a target locus by an unlinked silencing locus. HDGS can occur at the transcriptional level (transcriptional gene

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silencing; TGS) or at the post-transcriptional level (post-transcriptional gene silencing; PTGS). Founding papers that revealed these phenomena in transgenic plants did not identify the molecular mechanisms (Matzke et al. 1989; Linn et al. 1990; Napoli et al. 1990; Smith et al. 1990; van der Krol et al. 1990). Indeed, it took almost a decade to implicate double-stranded RNA (dsRNA) as the trigger of HDGS. It is now known that when a silencing locus produces dsRNAs corresponding to transcribed sequences of a target locus, HDGS occurs by PTGS (Waterhouse et al. 1998; Chuang and Meyerowitz 2000), which is similar to RNAi in animals (Fire et al. 1998). In contrast, when a silencing locus produces dsRNAs corresponding to promoter sequences of the target locus, HDGS occurs by TGS (Mette et al. 1999, 2000; Jones et al. 2001; Sijen et al. 2001). In plants, both dsRNA-induced TGS and PTGS result in methylation of homologous sequences at the target locus. Because methylation of promoter sequences is assumed to be responsible for dsRNA-induced TGS, it is often referred to as RNA-directed DNA methylation (RdDM). In contrast, methylation of transcribed sequences has no obvious impact on transcription (Elmayan et al. 1998). Nevertheless, mutations affecting methylation can impact maintenance and/or establishment of PTGS (Morel et al. 2000). PTGS results from target mRNA cleavage by the RNaseH activity of Argonaute (AGO) proteins that reside in RNA-induced silencing complexes (RISC; Tomari and Zamore 2005). mRNA cleavage in RISC is directed by short interfering RNAs (siRNAs) that derive from the processing of long dsRNAs that are complementary to target mRNAs by the RNase III Dicer. Mechanistically, PTGS is very similar to mRNA cleavage directed by endogenous miRNAs and trans-acting siRNAs (Vaucheret 2006).

The existence of distinct mechanisms for dsRNA-mediated TGS and PTGS in plants is supported by the involvement of distinct cellular components. Indeed, different members of the AGO, Dicer-like (DCL), and RNA-dependent RNA polymerase (RDR) families of proteins are required for the two processes. AGO1, DCL1, DCL4, and RDR6 act in the PTGS/miRNA/tasiRNA pathway (Peragine et al. 2004; Vaucheret et al. 2004; Vazquez et al. 2004; Gasciolli et al. 2005; Xie et al. 2005; Yoshikawa et al. 2005), whereas AGO4, DCL3, and RDR2 act in the TGS pathway (Zilberman et al. 2003; Chan et al. 2004; Xie et al. 2004). Some cellular components act in both TGS and PTGS pathways, although to different extents. For example, mutations in DDM1, MET1, NRPD1a, and RDR2 have a strong impact on TGS and a weak impact on PTGS (Morel et al. 2000; Herr et al. 2005). Reciprocally, AGO1 has a strong impact on PTGS and a weak impact on the taming of some transposons by TGS (Morel et al. 2002; Lippman et al. 2003). These results suggest that dsRNA-mediated TGS and PTGS likely result from the diversification of an ancient common mechanism. Another important difference between TGS and PTGS is the ability of viruses to counteract PTGS but not TGS. Most viruses encode proteins that can suppress various steps of PTGS, reflecting the role of PTGS as a natural antiviral defense mechanism (Vance and Vaucheret 2001; Roth et al. 2004; Dunoyer and Voinnet 2005). In contrast, no effect of viruses on TGS has been reported (Marathe et al. 2000; Mette et al. 2001).

A strict comparison of TGS and PTGS is difficult to achieve because it generally relies on the analysis of distinct silencing loci. A decade ago, we described a transgene locus that triggers both TGS and PTGS of different targets (Elmayan and Vaucheret 1996; Thierry and Vaucheret 1996). The 271 locus consists of multiple copies of the tobacco nitrite reductase (*NIR*) sequence in antisense orientation (*RIN*) driven by the Cauliflower Mosaic Virus (CaMV) 35S promoter and the bacterial neomycin phosphotransferase (*npt*) sequence driven by the CaMV 19S promoter. The 271 transgenic tobacco line was regenerated from kanamycin-resistant callus that spontaneously became sensitive to kanamycin and was unable to grow on medium containing nitrate or nitrite as the sole source of nitrogen (Vaucheret et al. 1992). The 271 locus was subsequently shown to silence, by TGS, any transgene driven by the 19S or 35S promoter and to silence, by PTGS, endogenous *NIR* genes as well as *NIR* transgenes (Elmayan and Vaucheret 1996; Park et al. 1996; Thierry and Vaucheret 1996), indicating that the 271 locus is a universal silencer of 19S, 35S, and *NIR* sequences. The 271 locus, however, was unable to silence *npt* transgenes by PTGS, but only by TGS when they are driven by the 19S or 35S promoter. The simultaneous triggering of TGS and PTGS by a single locus provided a unique system to study the similarities and differences between the two pathways. We previously reported that PTGS, but not TGS, is sensitive to infection by RNA viruses (Marathe et al. 2000). Here, we show that the 271 locus triggers TGS and PTGS through the production of dsRNAs corresponding to 19S, 35S, and *NIR* sequences, but not *npt*, explaining the silencing specificity of this locus. We describe mutants and epigenetic variants derived from the 271 locus, which allow us to propose a model for the production of dsRNAs from this locus.

Materials and methods

Transgenic tobacco lines

The *NIR* silenced lines 461-7 and 461-8, the β-glucuronidase (GUS)—silenced line 6b5 and GUS-expressing transgenic lines 23b1, 23b6, and 23b9 of tobacco (*Nicotiana tabacum* cv) have been previously described (Vaucheret et al. 1995a; Elmayan and Vaucheret 1996; Palauqui et al. 1997).

Grafting experiments and β-glucuronidase assay

Plants used for the grafting experiments were 9 weeks old. A wedge-grafting procedure was performed as previously described (Palauqui et al. 1997). Rootstocks were prepared by removing and discarding the top 5–6 cm of the plant. A vertical cut 1–2 cm long was made in the center of the stem. Scions were prepared by cutting the top 3–4 cm of the plant and trimming the bottom of the stem into a wedge. The graft junctions were secured using cotton string and parafilm. For the first week, the graft junction and scion were covered with plastic wrap and misted lightly with water to increase the humidity and prevent dehydration. RNA was isolated from both the rootstock and the scion plants immediately before grafting (pregraft samples). Approximately 4 weeks later, mid-size leaves were removed from the scions, and their GUS activity was monitored as previously described (Elmayan and Vaucheret 1996).

Southern-blot analysis

Genomic DNA analysis was performed as previously described (Elmayan and Vaucheret 1996). DNA samples (10 μg) were digested to completion with restriction enzymes, ethanol-precipitated, and resolved on 0.8% agarose gel. After depurination, the gel was blotted on nylon membranes (GeneScreenPlus, NEN^{TM} Life Science Products, Boston, MA, USA). The 32P labeled probe was synthesized by random priming. DNA gel blot hybridization and washing steps (30 min in 2× SSC, 0.1% SDS, and 5 min in 0.2× SSC, 0.1% SDS) were performed at 65°C.

Northern-blot analysis

Total RNA was prepared from leaves by phenol/chloroform extraction of samples ground to a fine powder in liquid nitrogen and allowed to thaw into five volumes of a buffer containing 100 mM Tris (pH 8), 100 mM LiCl, 10 mM EDTA (pH 8), 2% SDS. Ten micrograms per sample were separated electrophoretically on 1.5% formaldehyde-agarose gels and transferred on nylon membranes (Life Science Products) according to standard procedures. Equal loading was confirmed by visualizing the ethidium bromide-stained rRNA content under UV light after electrophoresis and then by hybridization of RNA filters with an actin-specific cDNA probe. cDNA fragments from the genes tested were 32P labeled with the Megaprime labeling kit (Amersham Biosciences Europe, Freiburg, Germany) according to the manufacturer's instructions and hybridized to the membranes. The riboprobe for the *NIR* antisens RNA detection was 32P-labeled and transcribed in the sense direction from *NIR* coding region according to the Ambion Megascript kit specification. RNA gel blot hybridization and washing steps (30 min in 2× SSC, 0.1% SDS, and 5 min in 0.2× SSC, 0.1% SDS) were performed at 65°C.

Double-stranded RNA and short interfering RNAs analyses

RNA was isolated essentially as described (Choi and Randles 1997). Leaves were ground in liquid nitrogen and the powder suspended in 3.5 ml RNA isolation buffer (100 mM Tris, pH 8.5, 100 mM NaCl, 20 mM EDTA, 1% sarkosyl) and 3.5 ml phenol/chloroform. Nucleic acids were precipitated with an equal volume of isopropanol. The pellet was resuspended in 2 ml 1.5× STE (1× STE = 100 mM Tris pH 7.5, 100 mM NaCl, 10 mM EDTA) after which 150 mg cellulose powder (MN 301, Macherey-Nagel) was added and mixed for 10 min at room temperature. One thousand and one hundred and sixty microliter of 96% ethanol was added in aliquots of 290 μl and mixed, resulting in a final mix of 1× STE/35% ethanol. Under these conditions single-stranded and dsRNA, but not DNA, bind to the cellulose. RNA was allowed to bind for 1 h. The cellulose was pelleted by a brief centrifugation, and washed three times with 1× STE/35% ethanol. RNA was eluted with 2 ml 1× STE, precipitated with ethanol, and resuspended in 400 μl H_2O. Residual cellulose was removed by a 1 min spin, the solution phenol/chloroform extracted, RNA again ethanol precipitated and finally dissolved in 10 mM Tris, 1 mM EDTA.

For nuclear and cytoplasmic RNA, leaves were ground in liquid nitrogen and transferred to 20 ml Hamilton buffer (10 mM Tris–HCl, pH 7.6, 1140 mM sucrose, 5 mM $MgCl_2$), supplemented with 2 mM of ribonucleoside-vanadyl complex as RNase inhibitor. A crude nuclear fraction was obtained by centrifugation at 1,000×*g* for 10 min. The supernatant was removed and cytosolic nucleic acids precipitated with ethanol.

RNA from the nuclear and cytosolic fractions was isolated by TRIzol (Gibco-BRL, Grand land, NY, USA) according to the manufacturers specifications. Any remaining genomic DNA was removed by the addition of 10 U DNase I (Boehringer, Indianapolis, IN, USA) incubated for 1 h in 10 mM Tris-HCl pH 7.9, 10 mM MgCl2, 50 mM NaCl, 1 mM DTT. RNA was recovered by phenol/chloroform extraction and ethanol precipitation. For each protection, 20 μg of nuclear RNA or 10 μg of cytoplasmic RNA was taken. To detect the protected fragments generated by the siRNAs, the products were electrophoresed on 12% polyacrylamide gels.

RNA probes were obtained by in vitro transcription using T3 and T7 RNA polymerase (Promega, Madison, WI, USA), and ^{32}P-UTP (Amersham) according to the manufacturers specifications. Appropriate DNA fragments were therefore cloned into plasmid pBS. The *NIR1* sense RNA probe extends from nt 1 to 307 of the *NIR* cDNA sequence; the 35S RNA probe extends from nt −200 to nt +20 relative to the transcription start site of the CaMV-35S promoter; the 19S probe was obtained from a 441 bp *Sma* I-*Xho* I fragment from the CaMV-19S promoter cloned into pBS. After in vitro transcription the DNA template was removed by DNase I and following a phenol extraction the RNA was precipitated with ethanol. To obtain the full-length probe the RNAs were size fractionated on a 7 M urea, 6% polyacrylamide gels, the band cut out and the RNA eluted in 500 mM NH_4Ac, 1 mM EDTA, 0.2% SDS, for 3–16 h. The probe was recovered by extraction with phenol/chloroform and ethanol precipitated using 40 μg yeast tRNA as a carrier.

For each protection, 20 μg of RNA was ethanol precipitated, dissolved in 4 μl H_2O, and mixed with 15 μl hybridization buffer (53 mM Pipes pH 6.4, 1.33 mM EDTA, 0.53 mM NaCl, 67% formamide). After adding the probe, 50–100 cps, the mixture was heated at 100°C for 1 min, and RNAs allowed to hybridize at 46°C for 16 h. An aliquot of 300 μl RNase digestion mix [20 μg RNaseA/ml (Boehringer), 8 U RNase T1/ml (Ambion, TX, USA), 10 mM Tris–HCl, pH 7.5, 5 mM EDTA, 300 mM NaCl] was added and the incubation continued for 1 h at 30°C. The digestion was stopped by adding 5 μl protease K (10 mg/ml) and 15 μl 10% SDS and an incubation at 37°C for 15 min. After phenol/chloroform extraction, nucleic acids were precipitated with ethanol using 10 μg tRNA as carrier. Pellets were resuspended in 6 μl H_2O and after the addition of 4 μl loading buffer (0.08% bromo phenol blue, 0.08% xylen cyanol, 20 mM EDTA in formamide) placed at 100°C for 1 min after which the samples were loaded on a 6% denaturing polyacrylamide gel.

To discriminate between single stranded RNA (ssRNA) and dsRNA, the RNA used for the protection assay was pretreated with 20 μg RNaseA/ml (Boehringer) at high-salt (10 mM Tris-HCl, pH 7.5, 5 mM EDTA, 0.3 M NaCl) or low-salt buffer (10 mM Tris–HCl, pH 7.5, 5 mM EDTA) for 30 min at 30°C in a volume of 300 μl. After a protease K digestion the RNA was recovered by phenol/chloroform extraction and ethanol precipitation and then used for the final RNase protection assay.

Results

35S-TGS, 19S-TGS, and *NIR*-PTGS triggered by the 271 locus correlate with the presence of 35S, 19S and *NIR* dsRNAs, and siRNAs.

Because the 271 locus is able to transcriptionally silence transgenes driven by the 35S promoter (Elmayan and Vaucheret 1996; Park et al. 1996), we looked for 35S RNAs aberrantly expressed from the 271 locus. RNase protection assays revealed two RNAs, 220-nt and 180-nt in length, corresponding to the 35S promoter (Fig. 1, upper panel, lane 2). To determine if these RNAs were single or double stranded, the RNAs were digested with RNaseA in high salt, which promotes cleavage of ssRNA but not dsRNA, or in low salt, which promotes cleavage of both ssRNA and dsRNA (Sijen and Plasterk 2003). Both the 220-nt and 180-nt 35S RNAs were resistant to RNaseA digestion in high salt (lane 3), but not in low-salt conditions (lane 4), indicating that these RNAs are likely double stranded. Because the 35S RNAs are double stranded, it is possible that they could be substrates for the RNAi pathway and be processed into siRNAs that could trigger transcriptional silencing of genes expressed under the control of 35S promoter, as previously observed in systems that produce dsRNAs from promoter sequences (Mette et al. 2000; Jones et al. 2001; Sijen et al. 2001). RNase protection assays of both nuclear and cytoplasmic RNA fractions revealed 35S-specific small RNAs in line 271 (Fig. 2). Although multiple RNAs ranging in size from 18-nt to 27-nt were present in both wild-type and 271 plants, at least one RNA species (indicated by a star in Fig. 2), specifically accumulated in line 271, indicating that this line produces 35S siRNAs, and is consistent with the ability of the 271 locus to trigger TGS of 35S-driven transgenes.

The 271 locus also triggers PTGS of endogenous *NIR* genes (Elmayan and Vaucheret 1996; Park et al. 1996). The 271 line does not accumulate mRNA from either of the four endogenous *NIR* genes or RNAs from the antisense transgene (35S-*RIN*) carried at the

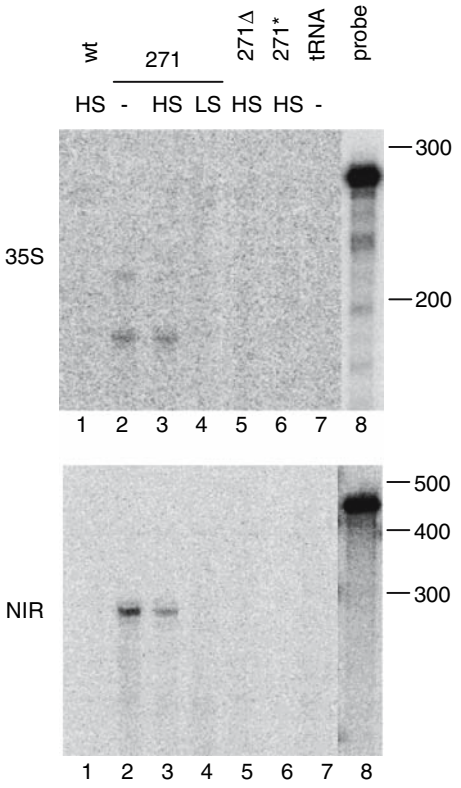

Fig. 1 The 271 locus produces dsRNA corresponding to 35S and *NIR* sequences. RNAs expressed from the 271 locus were analyzed by RNase protection assays without pre-treatment of the RNAs (−) or pre-digestion with RNaseA in high salt (*HS*), which cleaves ssRNA, not dsRNA, or in low salt (*LS*), which cleaves both ssRNA and dsRNA. 35S and *NIR* RNAs were resistant to RNaseA in HS, but not in LS, indicating that these RNAs are double stranded. No dsRNA was detected in the 271Δ mutant and in the 271* epigenetic variant

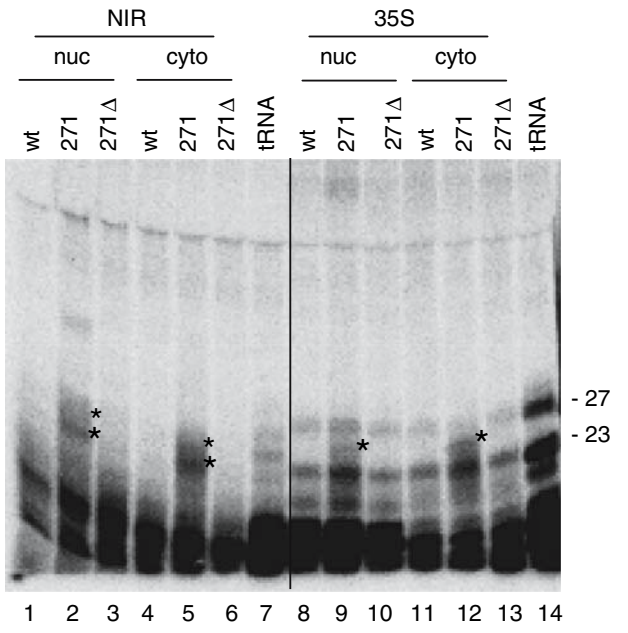

Fig. 2 The 271 locus produces siRNA corresponding to 35S and *NIR* sequences. Nuclear and cytoplasmic RNA fractions were analyzed by RNase protections. Although multiple RNAs ranging in size from 18-nt to 27-nt were present in both wild-type and 271 plants, one 35S-specific RNA and two *NIR*-specific RNAs (indicated by *stars*), specifically accumulated in line 271 but not in the 271Δ mutant

271 locus (Vaucheret et al. 1992). We detected antisense *NIR* RNAs in line 271 (Fig. 1, bottom panel, lane 2), consistent with the presence of the antisense 35S-*RIN* transgene. This transcript was detected after RNAseA digestion in high-salt condition (lane 3), but not after RNaseA digestion in low-salt condition (lane 4), suggesting that this RNA was double stranded. No *NIR* dsRNA was detected in wild-type plants (lane 1), indicating that the dsRNAs detected in line 271 likely derived from the 35S-*RIN* transgene. Nuclear and cytoplasmic RNA fractions of line 271 contained *NIR*-specific 21-nt to 25-nt small RNAs (Fig. 2, lanes 2 and 5), while *NIR*-specific small RNAs in this length range were not detected in wild-type plants (lanes 1 and 4). The presence of *NIR* long dsRNAs and siRNAs in line 271 is consistent with the degradation of endogenous *NIR* mRNAs by PTGS.

The 271 locus not only contains 35S and *RIN* sequences that trigger TGS of 35S-driven transgenes and PTGS of endogenous *NIR* genes, but it also contains 19S and *nptII* sequences. Similar to 35S-driven transgenes, 19S-driven transgenes are silenced by TGS by the 271 locus. However, in contrast to *NIR* endogenous genes, ectopic *nptII* transgenes are not silenced by the 271 locus, except when they are driven by the 35S or 19S promoter (Vaucheret 1993). RNase protection assays revealed two RNAs corresponding to the 19S promoter (data not shown), and these RNAs were resistant to RNaseA digestion in high salt, but not in low-salt conditions, indicating that these RNAs are also double stranded. Conversely, no antisense *nptII* transcripts were detected in RNA samples that contained *NIR* dsRNAs (data not shown), consistent with the absence of *nptII* silencing in line 271.

Post-transcriptional gene silencing but not transcriptional gene silencing induced by the 271 locus is graft-transmissible

Previous reports have indicated that PTGS results in the production of a graft-transmissible sequence-specific silencing signal (Palauqui et al. 1997; Voinnet et al. 1998; Crete et al. 2001; Mallory et al. 2003). To determine whether PTGS triggered by the 271 locus also results in the production of a systemic silencing signal, grafting experiments were performed using plant 271 as a silenced root-stock and 461-7/8 hybrids, which carry sense *NIR* transgenes that are efficiently silenced when grafted on silenced 461-8 rootstocks, as

non-silenced scions (Palauqui et al. 1997). *NIR* PTGS was triggered as efficiently in scions grafted on 271 as those grafted on 461-8 (Table 1), indicating that the 271 locus produces a systemic silencing signal. Because the *NIR* mRNA in the 461-7 × 461-8 scions is produced from a hybrid 35S:*NIR* promoter, it is possible that *NIR* PTGS and subsequent trans-silencing of the *NIR* promoter of the endogenous *NIR* genes in these scions could have been triggered by TGS of the 35S promoter. To determine if the 271 locus produces systemic silencing signals for TGS, scions carrying independent 35S-*uidA* (GUS) transgene loci that efficiently become silenced when crossed to line 271 were grafted on 271 or 271 × 35S-*uidA* rootstocks. No silencing of the 35S-*uidA* transgene was observed in any of the scions (Table 1), indicating that despite the involvement of dsRNAs and siRNAs in both TGS and PTGS triggered by the 271 locus, only PTGS spreads systemically.

A rearrangement of the 271 locus abolished transcriptional gene silencing and posttranscriptional gene silencing

To better understand the molecular basis for 271-mediated silencing, we searched for spontaneous mutants with impaired or modified silencing properties. To this end, a plant homozygous for the 271 locus was crossed with a plant homozygous for a target locus carrying a 19S-*nptII* transgene, which confers kanamycin resistance, and a 35S-*aux2* transgene, which confers sensitivity to NAM, a precursor of auxin. Progeny plants were kanamycin-sensitive because the 19S-*nptII* transgene is silenced in trans by the 271 locus, NAM-resistant because the 35S-*aux2* transgene is silenced in trans by the 271 locus, and *NIR*-deficient because the host *NIR* genes are silenced in trans by the 271 locus (data not shown). Protoplasts were prepared from the mesophyll of these hybrids, and plated on a medium supplemented with ammonium as a nitrogen source. One week later, 100,000 colonies were transferred to ammonium supplemented medium with kanamycin as a selective agent, and 100,000 colonies were transferred to ammonium supplemented medium without kanamycin. While 86,000 colonies grew on the medium devoid of kanamycin, only 12 colonies continued to grow, and form calli on the medium supplemented with kanamycin, indicating that either the 19S-*nptII* transgene carried by the 271 locus or the 19S-*nptII* transgene carried by the target locus was expressed. These 12 calli were transferred to fresh medium supplemented with ammonium and kanamycin where they developed shoots. These shoots were divided in two pieces, one piece was transferred to medium supplemented with ammonium and kanamycin, whereas the other was transferred to medium supplemented with ammonium and NAM. The 12 shoots rooted on kanamycin-containing medium, indicating that the 19S-*nptII* transgene carried by the 271 locus or the target locus or both was expressed in these 12 plants. In addition, growth of the 12 duplicate shoots was inhibited on NAM, indicating that the 35S-*aux2* transgene carried by the target locus was expressed. When transferred to the greenhouse and watered with nitrate, the 12 kanamycin-resistant plants continued to develop normally, indicating that the host *NIR* genes were expressed.

Table 1 PTGS but not TGS is graft-transmissible

Scion	Gene (State[a])	Rootstock	Gene (State[a])	No. of grafts	No. of silenced scions
461-7 × 461-8	*NIR* (NS)	PBD6	WT	10	0
461-7 × 461-8	*NIR* (NS)	461-8	*NIR* (PTGS)	20	20
461-7 × 461-8	*NIR* (NS)	271	*NIR* (PTGS)	20	20
461-7 × 461-8	*NIR* (NS)	271 × 23b1	*NIR* (PTGS)	5	5
461-7 × 461-8	*NIR* (NS)	271 × 23b6	*NIR* (PTGS)	5	5
461-7 × 461-8	*NIR* (NS)	271 × 23b9	*NIR* (PTGS)	5	5
PBD6 × 23b1	35S-*uidA* (NS)	PBD6	WT	5	0
PBD6 × 23b1	35S-*uidA* (NS)	6b5	35S-*uidA* (PTGS)	5	5
PBD6 × 23b1	35S-*uidA* (NS)	271 × 23b1	35S-*uidA* (TGS)	20	0
PBD6 × 23b6	35S-*uidA* (NS)	PBD6	WT	5	0
PBD6 × 23b6	35S-*uidA* (NS)	6b5	35S-*uidA* (PTGS)	5	5
PBD6 × 23b6	35S-*uidA* (NS)	271 × 23b6	35S-*uidA* (TGS)	20	0
PBD6 × 23b9	35S-*uidA* (NS)	PBD6	WT	5	0
PBD6 × 23b9	35S-*uidA* (NS)	6b5	35S-*uidA* (PTGS)	5	5
PBD6 × 23b9	35S-*uidA* (NS)	271 × 23b9	35S-*uidA* (TGS)	20	0

[a] *NS* non-silenced. NS transgenic scions were grafted onto wild-type plants (*WT*) or transgenic plants carrying an homologous transgene silenced by TGS or PTGS. PBD6 is the wild-type tobacco cultivar. 461-7 is a 35S-*NIR* line that does not trigger PTGS spontaneously. 461-8 is a 35S-*NIR* line that triggers PTGS spontaneously. 461-7 × 461-8 hybrids do not trigger PTGS spontaneously. 23b1, 23b6, and 23b9 are 35S-*uidA* lines that do not trigger PTGS spontaneously. 6b5 is a 35S- *uidA* line that triggers PTGS spontaneously

DNA was extracted from the 12 plants and subjected to Southern-blot analysis using *aux2* and 271 specific probes. As expected from the results of growth inhibition by NAM, the target locus was present in all plants. In contrast, sequences homologous to the 271 locus were present in only one plant (data not shown), indicating that the 271 locus is lost in somatic cells at a frequency of 1.3×10^{-4}, similar to the frequency of loss in meiotic cells reported previously (Vaucheret et al. 1995b). To further characterize the plant that had retained 271 sequences, the target locus was segregated away. Using a combination of restrictions enzymes and probes, DNA-blot analysis revealed either the absence of a band or a shift in mobility (Fig. 3), suggesting that a deletion had occurred within the 271 locus. This rearranged locus was therefore named 271Δ. Plants carrying both the 271Δ and target loci were kanamycin resistant, whereas plants carrying only the 271Δ locus were kanamycin sensitive, indicating that the kanamycin resistance was due to the expression of the 19S-*nptII* transgene from the target locus and not the 271Δ locus. The ability of plants carrying the 271Δ and target loci to grow on nitrate and to express the 19S-*nptII* and 35S-*aux2* transgenes carried by the target locus suggested that the 271Δ locus is unable to trigger PTGS or TGS in trans. To test this hypothesis, independent transgenic lines carrying 19S-*nptII*, 35S-*hpt* (hygromycin resistance), or 35S-*uidA* (GUS) transgenes were crossed with a plant homozygous for the 271Δ locus. Control crosses were done with plant 271 and with a wild-type plant. As expected, 19S-*nptII*, 35S-*hpt*, and 35S-*uidA* hybrids carrying the 271 locus were kanamycin-sensitive, hygromycin-sensitive, or GUS-negative, respectively. In contrast, hybrids carrying the 271Δ locus were kanamycin-resistant, hygromycin-resistant, or GUS-positive. In addition, hybrids carrying the 271 locus were unable to grow on nitrate, whereas hybrids carrying the 271Δ locus grew normally on nitrate. In addition, *NIR* and 35S dsRNAs or siRNAs could not be detected in line 271Δ (Figs. 1, 2), which likely explains the absence of TGS and PTGS in this line.

The results described above strongly suggest that the 271Δ locus derives from the 271 locus by a deletion that prevents this locus from producing dsRNAs and siRNAs and subsequently impairs TGS and PTGS. However, other hypotheses that could explain the impairment of the 271 locus silencing properties, such as 271 locus translocation to another chromosome, are possible. To test if the 271Δ locus was located at the same chromosomal position as the 271 locus, i.e., if these two loci are allelic, a plant homozygous for the 271Δ locus was crossed with a plant homozygous for the 271 locus and the 271/271Δ hybrid was backcrossed to wild-type plants. If the 271Δ locus was carried on a different chromosome than the 271 locus, 25% of the plants would lack 271 sequences. Conversely, if the 271Δ locus was allelic to the 271 locus, all plants should carry 271 sequences. DNA was extracted from 48 plants, and the presence of 271 sequences was tested by PCR. All 48 plants analyzed contained 271 sequences, strongly suggesting that the 271Δ locus is allelic to the 271 locus and derives from the 271 locus by a simple local DNA rearrangement, not a chromosomal translocation.

We also tested whether the 271Δ locus could be subjected to paramutation, i.e., if it could acquire all of or part of the silencing properties of the 271 locus after being exposed to inter-allelic interaction with the 271 locus. The 271/271Δ hybrid was crossed with a target line homozygous for a 35S-*uidA* transgene and the seeds were sown on medium supplemented with nitrate as the sole source of nitrogen. About 50% (49/96) of the plants were able to grow on nitrate, suggesting that the 271Δ locus cannot acquire the PTGS properties of the 271 locus. Seedlings were also stained for GUS activity and ~50% (47/96) of the plants showed GUS silencing, suggesting that the 271Δ locus cannot acquire the TGS properties of the 271 locus. Together, these results suggest that the 271Δ locus cannot be subjected to paramutation by the 271 locus, and that the DNA rearrangement that led to the formation of the 271Δ locus irreversibly eliminated the ability to trigger TGS and PTGS in trans.

The 271 locus can exist in a non-silent epigenetic state

The accumulation of *NIR*, 19S and 35S dsRNAs, and siRNAs is compromised in 271Δ plants, suggesting that the integrity of the 271 locus is critical for their accumulation. To better understand the molecular basis for 271-triggered TGS and PTGS, we searched for additional plants with impaired or modified silencing properties. Fifty thousand seeds obtained by self-fertilization of a plant homozygous for the 271 locus were sown on medium supplemented with kanamycin. None of the seedlings were resistant to kanamycin. Because loss of silencing in a rearranged allele could be masked by trans-silencing mediated by an unaffected allele, additional selections were carried out with F1 seeds obtained by crossing plant 271 with a wild-type plant. Only one kanamycin-resistant plant was recovered out of 50,000 F1 seeds. When transferred to soil and supplemented with nitrate, this plant was able to grow normally, indicating that *NIR* PTGS was impaired in this plant. DNA blot analysis indicated that the 271 locus was present without obvious

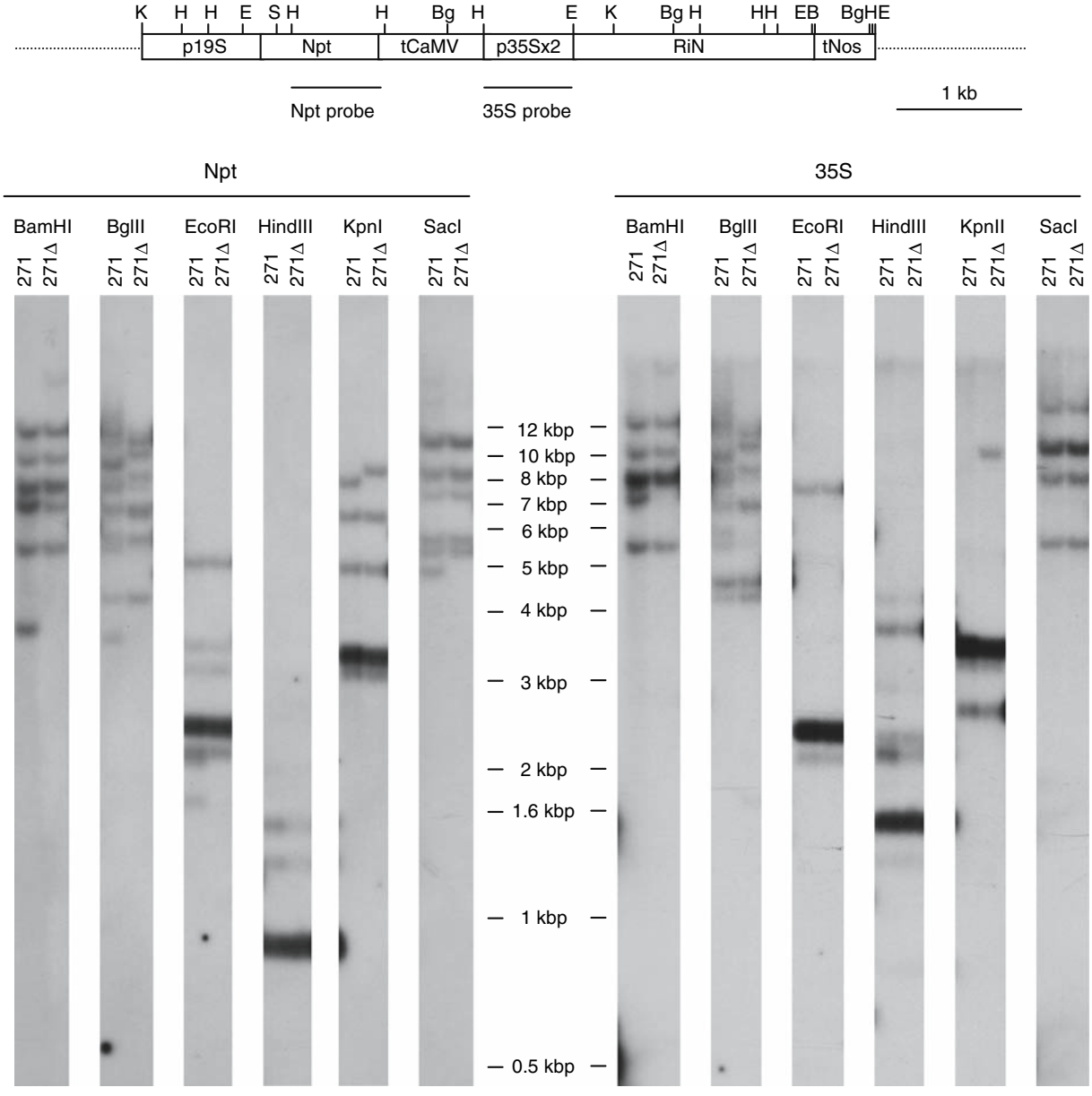

Fig. 3 The 271Δ locus consists in a rearrangement of the 271 locus. DNA extracted from 271 and 271Δ plants was digested by a combination of restriction enzymes and hybridized with a 35S probe (*left*) or a *nptII* probe (*right*). The absence of restriction fragments in 271Δ suggests that a small deletion had occurred within the 271 locus. The shift of a *Kpn* I fragment in plant 271Δ suggests that the deletion also affects part of the genomic adjacent sequences. The restriction map of the 35S-*RIN* and 19S- *nptII* transgenes carried by the pRIN plasmid and the position of the probes are shown on the top panel. B: *Bam* HI, Bg: *Bgl* II, E: *Eco* RI, H: *Hind* III, K: *Kpn* I, S: *Sac* I

rearrangement (data not shown). These results suggested either that this plant contained a dominant suppressor of TGS and PTGS in a locus other than 271 or carried an epigenetic variant form of the 271 locus (called 271*) or carried a mutation at the 271 locus that cannot be detected by southern blot.

To discriminate among these three hypotheses, this plant (which was hemizygous for the transgene locus) was selfed and sown on either a medium supplemented with kanamycin and ammonium as a source of nitrogen, or a medium supplemented with nitrate as a source of nitrogen. If this plant contained a dominant suppressor of TGS and PTGS in a locus other than 271, 56.25% (9/16) of the plants should be kanamycin-resistant and 81.25% (13/16) should grow on nitrate. If it carried a mutation at the 271 locus or a stable epigenetic variant form of the 271 locus, 75% of the plants should be kanamycin-resistant and 100% of plants

should grow on nitrate. However, if the epigenetic variant form is unstable, the percentage of kanamycin-resistant plants should be lower, and the percentage of plants unable to grow on nitrate should be high. Only 1.5% (10/1567) of the plants were able to grow on kanamycin. This very low-percentage excluded the hypothesis of a dominant mutation suppressing TGS and PTGS at the 271 locus or at an unlinked locus. Rather, it suggested this plant carried an epigenetic variant form of the 271 locus (271*), which spontaneously reverts to the original 271 form at a high-frequency during meiosis. Consistent with this hypothesis, approximately 75% (147/200) of the plants were unable to grow on nitrate. In addition, a strict correlation between kanamycin resistance (conferred by the 271* form) and the loss of *NIR* PTGS was observed, as all kanamycin-resistant plants were able to grow normally when transferred to medium supplemented with nitrate. DNA digestion with the methylation-sensitive enzyme *Msp* I revealed a decrease in *npt* DNA methylation in all kanamycin-resistant plants, although to various extents (Fig. 5a), consistent with the instability of the 271* locus. The ten kanamycin-resistant plants were selfed and seeds were sown on medium containing nitrate or kanamycin. As observed for the progeny of the original plant 271*, 1–2% of each of the ten progenies grew on kanamycin. In addition, 25% of each progeny grew on nitrate, indicating that the ten kanamycin-resistant plants were hemizygous for the 271* locus. This result suggests that the 271 locus can be maintained in the 271* form at low frequency and in a hemizygous state.

To analyze the other silencing properties of the 271* locus, a kanamycin-resistant plant hemizygous for the 271* locus was crossed with a transgenic line homozygous for a 35S-*hpt* transgene, and the progeny seeds were sown on medium supplemented with hygromycin. Approximately 50% (198/407) of the plants were hygromycin-sensitive, which was expected based on the ability of the 271* locus to revert to the original 271 form at high frequency. To determine if the plants that inherited the 271* variant form were able to trigger TGS, seeds were first selected on a medium supplemented with kanamycin to identify the plants carrying the 271* variant locus. As expected, approximately 1% (7/672) of the seeds was able to grow on kanamycin. These seven plants were then transferred to medium supplemented with hygromycin. All kanamycin-resistant plants were able to grow on hygromycin, indicating that the 271* variant form is unable to trigger TGS. When transferred to soil, these plants also were able to grow on nitrate, confirming that the epigenetic variant locus 271* does not exhibit any of the silencing properties of the 271 original locus. To determine the cause of this impairment, line 271* was examined for dsRNAs as described above. *NIR*, 35S, or 19S dsRNAs could not be detected (Fig. 1, lane 6), indicating that the silencing properties of the 271 locus do not depend on the structure of the locus only, but also on its epigenetic state.

The 271Δ locus can exist in three epigenetic states

The identification of the 271* variant locus prompted us to analyze the epigenetic stability of the 271Δ locus. Although the 271Δ locus appears to carry an intact 19S-*npt* transgene (Fig. 3), the 271Δ plant was not able to grow on kanamycin. In addition, 10,000 seeds deriving from the self-fertilization of a plant homozygous for the 271Δ locus were unable to grow on kanamycin, suggesting either that the 19S-*npt* transgene was silenced in cis or that mutations occurred in every copy of the 19S-*npt* transgene during the rearrangement that led to the formation of the 271Δ locus. To distinguish between these two possibilities, a plant homozygous for the 271Δ locus was crossed to a wild-type plant and 50,000 F2 seeds were plated on medium supplemented with kanamycin. Fifteen kanamycin-resistant plants were recovered, indicating that the 19S-*npt* transgene was functional but silenced in cis in plant 271Δ. Among these 15 plants, 11 plants (referred to as 271Δ*) were able to grow on nitrate and exhibited leaves as green as wild-type plants and 271Δ plants, indicating that the endogenous *NIR* genes were expressed, whereas four plants (referred to as 271Δ**) were also able to grow on nitrate but exhibited mild chlorosis (Fig. 4), which is symptomatic of a partial deficiency in *NIR*.

The methylation status of the 271Δ locus in representative 271Δ* and 271Δ** plants was compared with that of the original 271Δ plant by DNA blot analysis using methylation-sensitive enzyme *Msp* I. The 271Δ locus in the original 271Δ plant was methylated at a level similar to that of the original 271 locus (Fig. 5b), consistent with the silent state of the 19S-*npt* and 35S-*RIN* transgenes in both the 271 and 271Δ plants. In contrast, 271Δ* and 271Δ** plants showed a reduction in DNA methylation (Fig. 5b), consistent with the expression of the 19S-*npt* transgene in these plants. Methylation was more reduced in 271Δ** plants compared with 271Δ* plants (Fig. 5b), which could result in the expression of the 35S-*RIN* transgene in addition to the 19S-*npt* transgene, and to the production of *RIN* antisense RNA that could be responsible for the mild chlorosis observed in 271Δ** plants. Indeed, Northern-blot analysis using sense and antisense *NIR* probes revealed that, like 271Δ, 271*, and wild-type plants,

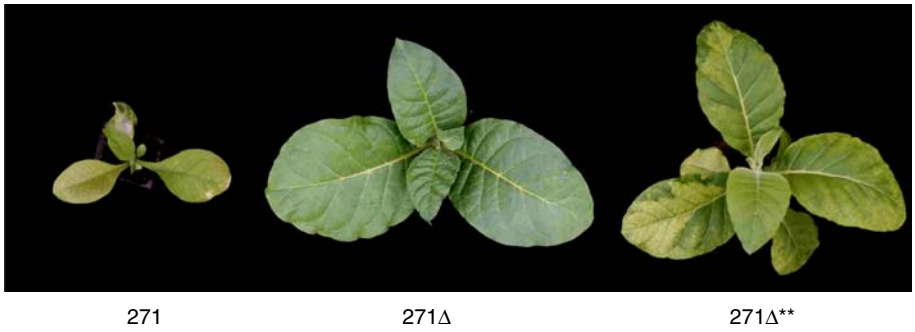

Fig. 4 Differences in leaf chlorosis between plant 271 and the epigenetic variant 271Δ**. Plants were watered with a nutrient solution containing nitrate as sole source of nitrogen. Plant 271 remains dwarf and exhibits a strong chlorosis indicative of the complete inhibition of the endogenous *NIR* genes. The deletion mutant 271Δ and the epigenetic variant 271Δ* cannot be distinguished from a wild-type plant, indicating a complete functioning of the endogenous *NIR* genes. The epigenetic variant 271Δ** grows as well as plants 271Δ and 271Δ* but exhibit a mild leaf chlorosis, indicative of a partial functioning of the endogenous *NIR* genes

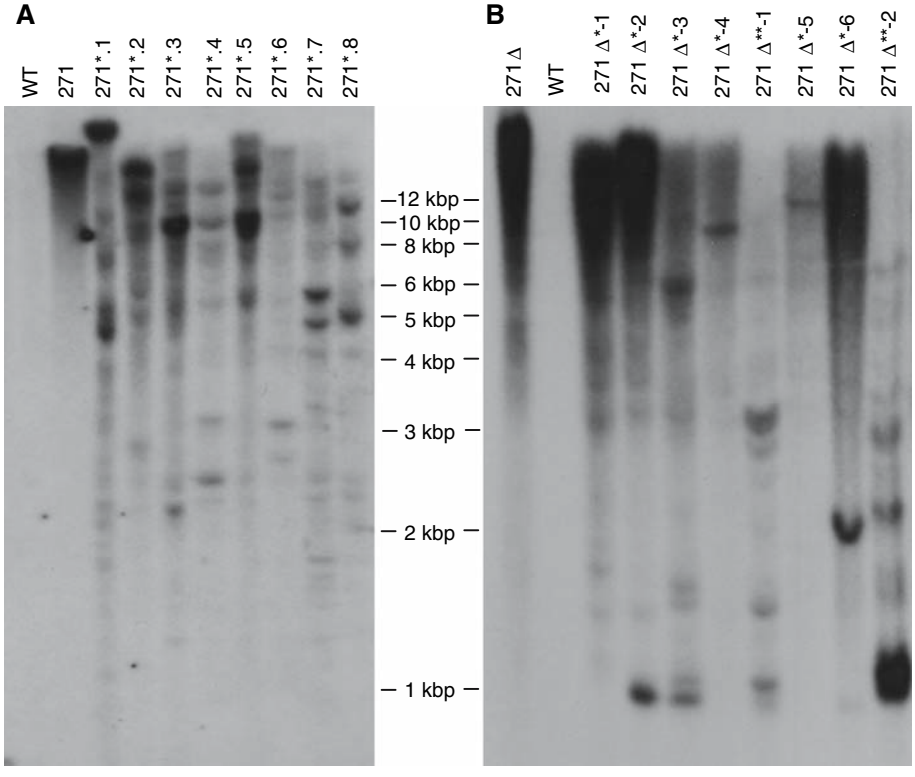

Fig. 5 Reactivation of the 19S-*nptII* transgene in 271 and 271Δ epigenetic variants correlates with decreased DNA methylation. **a** DNA extracted from plant 271 and eight plants derived from the 271* epigenetic variant by self-fertilization was digested by the methylation-sensitive enzyme *Msp* I and hybridized with a *nptII* probe. A single band of ca 50 kb is observed in plant 271, indicative of the complete methylation of the locus. Bands of lower, although variable, molecular weight are observed in plants derived from the 271* epigenetic variant, indicating both the decreased methylation of the 271* form of the locus and of its propensity to spontaneously revert to the silent 271 form. **b** DNA extracted from 271Δ, 271Δ*, and 271Δ** plants was digested by the methylation-sensitive enzyme *Msp* I and hybridized with a 19S probe. A single band of ca 50 kb is observed in plant 271, indicative of the complete methylation of the locus. Limited or strong decrease in DNA methylation are observed in 271Δ* and 271Δ** epigenetic variants, respectively

271Δ* plants accumulated sense *NIR* mRNA but not *RIN* antisense RNA whereas 271Δ** plants accumulated antisense *RIN* RNA and had reduced accumulation of endogenous *NIR* mRNA compared with wild-type plants, 271Δ or 271Δ* plants (Fig. 6). These results indicate that 271Δ** plants express the 35S-*RIN* transgene, which may partially inactivates the endogenous *NIR* genes by antisense silencing.

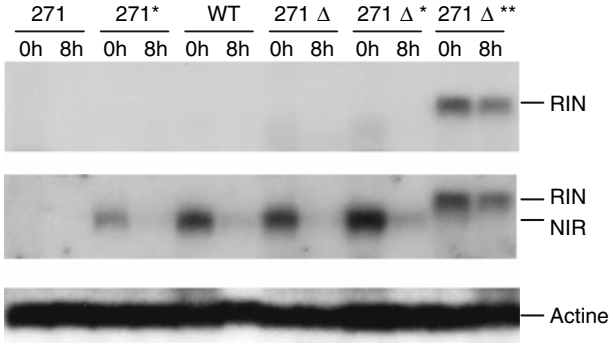

Fig. 6 Mutants and epigenetic variants derived from 271 accumulate endogenous *NIR* and/or antisense *RIN* transcripts. RNA was extracted from wilt-type (*wt*), 271, 271*, 271Δ, 271Δ*, and 271Δ** plants at the beginning of the light period (0 h) or after 8 h of illumination and hybridized with a single-stranded probe complementary to the antisense *RIN* transcript (*upper panel*). The blot was re-hybridized with a single-stranded probe complementary to the sense *NIR* transcript without preliminary stripping (*middle panel*). The blot was stripped and re-hybridized with an *actin* probe as a loading control (*lower panel*). The endogenous *NIR* transcript, which is subjected to a circadian regulation, is detectable at the beginning of the day period in wild-type, 271*, 271Δ, 271Δ*, and 271Δ** plants, consistent with their ability able to grow on nitrate. The antisense transcript, driven by the constitutive 35S promoter, is detectable at both the beginning and the middle of the day period in 271Δ ** plants, consistent with their chlorotic phenotype

Discussion

The transgenic tobacco line 271 is unique in that a single transgene locus triggers both TGS and PTGS (Vaucheret 1993; Elmayan and Vaucheret 1996; Park et al. 1996). TGS affects unlinked transgenes driven by promoters sharing homology with 271 promoter sequences while PTGS affects unlinked endogenous genes or transgenes expressing mRNAs sharing homology with 271 coding sequences. For years, the molecular basis of this unique silencing phenomenon has remained undetermined. However, based on the current knowledge on transgene-induced TGS and PTGS, the 271 locus was expected to produce dsRNAs corresponding to the trans-silenced sequences, which would be subsequently processed into siRNAs that eventually trigger TGS when homologous to promoter sequences or PTGS when homologous to coding sequences. Here we validate this hypothesis by showing the accumulation of dsRNAs corresponding to 19S, 35S, and *NIR* sequences of the 271 locus that silences 19S- and 35S-driven transgenes as well as *NIR*-encoding endogenous genes and transgenes. In contrast, we could not detect dsRNAs corresponding to the *nptII* sequences of the 271 locus that is unable to silence unlinked *nptII* transgenes, except when they are driven by a 19S or 35S promoter. We also detected siRNAs corresponding to 35S and *NIR* sequences, strongly suggesting that siRNAs are the actual molecules that trigger TGS and PTGS on homologous sequences.

The 271 locus consists of the complex insertion of six to seven copies of the 35S-*RIN* and 19S-*nptII* transgenes. Because transformation was achieved by protoplast electroporation using naked DNA and not by *Agrobacterium*-mediated T-DNA transfer, we were not able to resolve the exact structure of the 271 locus. It is unlikely that independent dsRNAs are produced from 19S, 35S, and *NIR* sequences. Rather, it is probable that 35S, *NIR*, and 19S sequences, but not *nptII*, are transcribed from an endogenous promoter located at one end of the locus (Fig. 7), resulting in the production of a chimeric 19S-*RIN*-35S aberrant RNA that could be converted into dsRNA by a cellular RDR similar to RDR6 that is involved in S-PTGS in Arabidopsis. Alternatively, an endogenous promoter located at one end of the locus could transcribe an inverted repeat of 35S, *RIN*, and 19S sequences, resulting in the direct production of dsRNAs. We favor the hypothesis of an endogenous promoter adjacent to transgene sequences rather than a promoter internal to the transgene locus (19S or 35S) because the 271 locus exhibits dense methylation, presumably resulting from RdDM mediated by 271-dsRNAs and/or 271-siRNAs. RdDM likely results in TGS of the 19S and 35S promoters within the 271 locus, but not of the putative adjacent plant promoter, which therefore could continuously transcribe sequences within the methylated 271 locus. Indeed, previous run-on analyses on methylated versus non-methylated transgenes have revealed that methylation of transcribed sequences does not affect the rate of transcription (Elmayan et al. 1998). However, in one case, methylation has also been shown to spread from coding to promoter sequences, resulting in TGS (Van Houdt et al. 2003). It is conceivable that such a spread of methylation from the 271 transgene sequences into the adjacent plant promoter has happened in the epigenetic variant (271*) that we recovered by plating 50,000 hemizygous 271 seeds on a medium supplemented with kanamycin. This plant showed reduced methylation of the transgene sequences, which likely results from the maintenance of symmetric methylation in the absence of 271-siRNAs. Indeed, no dsRNAs or siRNAs were detected in plant 271*, which is likely the reason why it was unable to trigger TGS or PTGS in trans. However, in the absence of de novo production of 271-siRNAs supporting dense methylation within the 271 transgene sequences, the spread of methylation into the adjacent plant promoter is unlikely to be maintained, resulting in the reactivation

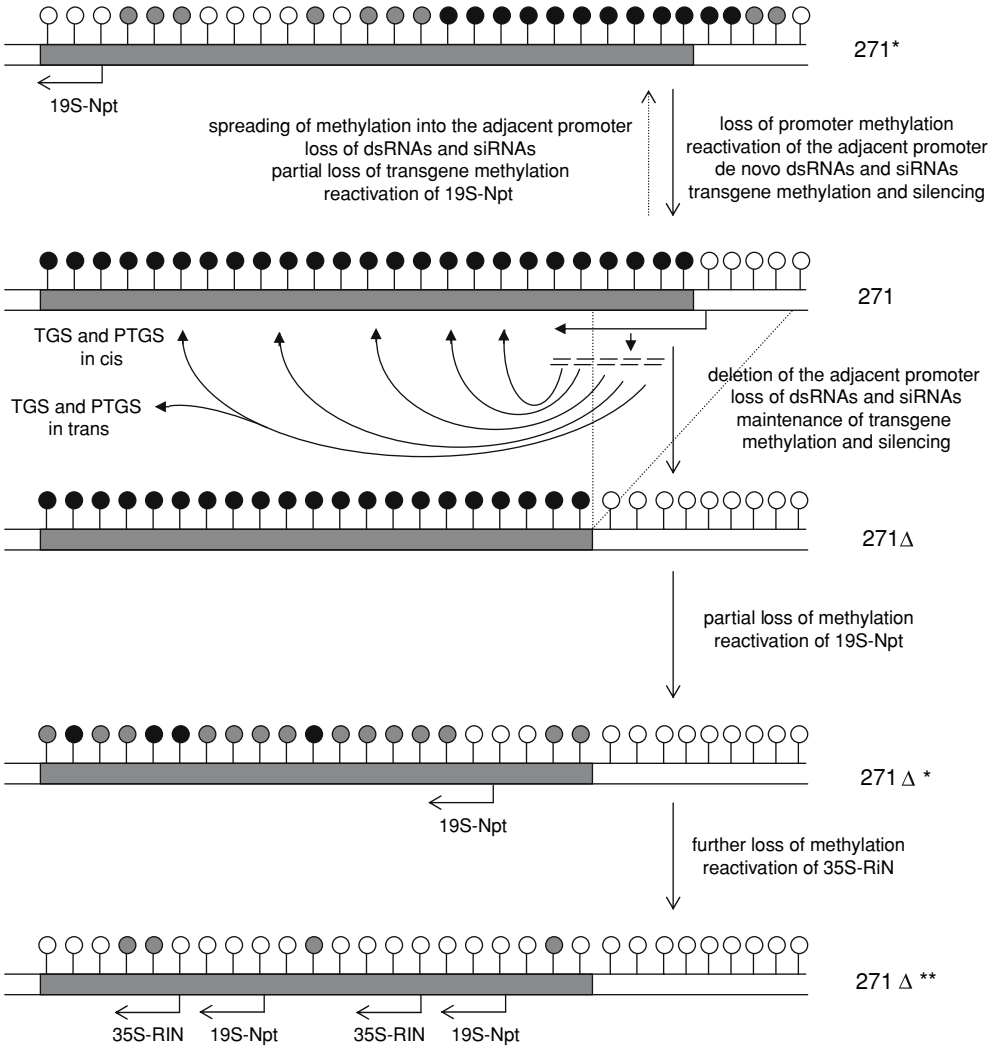

Fig. 7 Hypothetical model for the silencing properties of 271 and 271-derived mutants and epigenetic variants. Transgene and genomic sequences at the 271 locus are represented as hatched and white boxes, respectively. High, intermediate, and low levels of methylation are represented by *black, gray* and *white circles*, respectively. The 271 locus (second panel from the top) consists in the crumbled insertion of 6–7 copies of a plasmid carrying the 35S-*RIN* and 19S-*nptII* transgenes. It is likely that 35S, *NIR*, and 19S sequences, but not *npt*, are transcribed from an endogenous promoter located at one end of the locus, resulting in the production of 35S, 19S, and *RIN* dsRNAs that are processed into siRNAs that triggers silencing and methylation of homologous sequences in cis and in trans. Methylation of the transgene sequences could accidentally spread into the endogenous promoter and trigger transcriptional silencing (*top panel*: epigenetic variant 271*). No dsRNAs or siRNAs are detected in 271*, which is likely the reason why the locus no longer triggers TGS and PTGS and shows reduced methylation. As a result, the adjacent plant promoter is reactivated, leading to de novo production of 271-dsRNAs and 271-siRNAs and reversion to the silent 271 state at high frequency. A small deletion of a portion of the 271 locus and of the adjacent endogenous promoter in plant 271Δ (*third panel* from the *top*) irreversibly abolishes the production of dsRNAs and siRNAs, which is likely the reason why the locus is unable to trigger TGS or PTGS in trans. The 271Δ locus still exhibits dense methylation, presumably through the maintenance of symmetric methylation inherited from the original 271 locus. This high level of methylation is likely responsible for the silencing of the 35S-*RIN* and 19S-*nptII* transgenes at the 271Δ locus. Epigenetic variants that re-expressed either the 19S-*nptII* transgene alone (*fourth panel* from the *top*) or both the 19S-*nptII* and 35S-*RIN* transgenes (*fifth panel* from the *top*) were identified in an outcross of plant 271Δ. Transgene reactivation correlated with demethylation, supporting the hypothesis that methylation inherited from the original 271 locus was responsible for the silencing of transgenes at the 271Δ locus

of the plant promoter and to the de novo production of 271-dsRNAs and 271-siRNAs. Consistent with this hypothesis, we observed that the 271 locus spontaneously reverted from an active (271*) state to a silent (271) state with a frequency of 99% at each meiosis. Therefore, the silent state could be considered as the natural state of the 271 locus.

Our attempts to isolate mutants that have irreversibly lost the trans-silencing properties of the 271 locus resulted in the identification of a plant that has lost a

portion of the 271 locus. This plant, referred to as 271Δ does no longer produce dsRNAs or siRNAs, which is likely the reason why it is unable to trigger TGS or PTGS in trans. The deletion in plant 271Δ is likely to affect transgene sequences adjacent to the plant promoter that is responsible for the production of dsRNAs and siRNAs. Indeed, the loss of trans-silencing properties could not be reverted either spontaneously or after exposure to the original 271 locus, indicating that the 271Δ is unable to undergo paramutation. The 271Δ locus, nevertheless, exhibits dense methylation, which likely is responsible for maintaining the 35S-*RIN* and 19S- *nptII* transgenes in silenced states. However, this silent state is unlikely to represent the most natural epigenetic state of the 271Δ locus. Indeed, epigenetic variants that expressed either the 19S-*nptII* transgene (271Δ*) or both the 19S-*nptII* and 35S-*RIN* transgenes (271Δ**) were identified by plating seeds derived from the out-cross of plant 271Δ on a medium supplemented with kanamycin. Transgene reactivation correlated with demethylation, similar to that observed in the 271* epigenetic variant. However, 271Δ* and 271Δ** epigenetic variants were recovered at much higher frequency than 271* epigenetic variants, indicating that silencing and methylation were more easy to erase from the 271Δ locus than from the 271 locus. In addition, transgene reactivation was maintained in the progeny of 271Δ* and 271Δ** plants whereas silencing was re-established at a very high frequency in the progeny of 271* plants. These results suggest that the natural state of the 271 locus is silent and methylated whereas the natural state of the 271Δ locus is active and unmethylated. We propose that the dense methylation observed in the original 271Δ plant had been inherited from the methylated 271 locus because of the absence of selection pressure for reactivation of the transgene carried by the 271Δ locus. Indeed, the original 271Δ plant was resistant to kanamycin because the 271Δ locus was unable to silence an unlinked 19S-*nptII* transgene in *trans*, and not because the 19S-*nptII* transgene carried by the 271Δ locus was expressed. This contrasts to the 271* epigenetic variant, which was obtained by selection pressure for reactivation of the transgene carried by the 271 locus. Only subsequent selection pressure for reactivation of the 19S-*nptII* transgene carried by the 271Δ locus led to the stabilization of the 271Δ locus in an active state, which likely reflects the natural state of a locus that does not produce dsRNA.

The 271 locus allowed us to dissect and compare the properties of TGS and PTGS, owing to the simultaneous production of promoter sequence siRNAs and coding sequence siRNAs by a single locus. We took advantage of this system to examine two aspects of silencing: the response to virus infection and the propagation of silencing signals. Infection of plant 271 by RNA viruses counteracted PTGS but not TGS (Marathe et al. 2000), a result that may be explained by the fact that RNA viruses replicate in the cytoplasm where they have the potential to interfere with PTGS, but not with TGS, which presumably occurs exclusively in the nucleus (Marathe et al. 2000; Mette et al. 2001). Grafting experiments also revealed that PTGS, but not TGS, is graft-transmissible. Several hypotheses could account for the absence of systemic TGS. One hypothesis is that TGS-associated dsRNAs and siRNAs are confined to the nucleus and thus are unable to move from cell to cell and into the phloem, which requires that they first move from the nucleus to the cytoplasm. However, since TGS and PTGS in plant 271 likely result from the production of a common dsRNA, it is difficult to explain why PTGS-associated dsRNAs and siRNAs could be exported to the cytoplasm whereas TGS-associated dsRNAs and siRNAs would be retained in the nucleus. Therefore, a second hypothesis that could account for the absence of systemic TGS is that TGS-associated dsRNAs or siRNAs can move but that they are not amplified and thus cannot trigger systemic TGS because the amount of molecules that enter into recipient cells is too low. If the degree of amplification depends on the amount of target molecules, highly transcribed mRNAs, for example, those deriving from 35S-driven transgenes, will be prone to trigger systemic PTGS, whereas promoters that are targeted by siRNAs will not be able to trigger systemic TGS because promoters are ordinarily not transcribed into RNA or are transcribed at low levels. A third possibility is that TGS-associated dsRNAs and siRNAs are not the systemic silencing signal and that the signal is produced downstream of any of the steps that TGS and PTGS have in common. Thus far, an universal silencing locus such as the 271 locus has only been identified in tobacco, which is an amphitetraploid species, strongly compromising genetic studies and mutant isolation. However, the recent progress in the identification of genes that control silencing in Arabidopsis will certainly help at designing gain-of-function or loss-of-function transgene constructs that could potentially modify the silencing machinery in plant 271 and provide further insights in our understanding of the similarities and differences between TGS and PTGS mediated by dsRNAs.

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